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Structural Effects of *N*-Arylcarbamoylpyrazolines on Calcium Uptake in Rat Brain Synaptosomes

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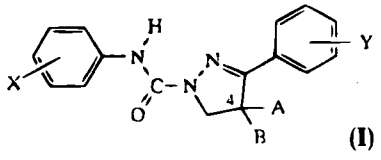
Abstract: *N*-Arylcarbamoylpyrazolines with various substituents at the *para* position of the carbamoyl benzene ring inhibited ATP-dependent Ca^{2+}-uptake in synaptosomes prepared from the rat brain. The activity of these compounds was evaluated as $\log(1/I_{50})$, the reciprocal logarithm of half inhibitory concentration, I_{50} (M), from the concentration–response curve for the inhibition of Ca^{2+}-uptake. Among the compounds tested, methyl 3-(4-chlorophenyl)-4-methyl-1-[*N*-(4-trifluoromethylphenyl)carbamoyl]-2-pyrazoline-4-carboxylate was the most potent, the I_{50} value of which as $9{\cdot}12 \times 10^{-7}$ M. Variations in the activity in terms of $\log(1/I_{50})$ were quantitatively analysed using a substituent parameter, showing that the higher the electron-withdrawing effect of the substituent, the higher was the activity. The substituent effects were similar to those on insecticidal activity against the Americal cockroach. The higher the inhibitory activity against Ca^{2+} uptake, the higher seemed to be the insecticidal activity. Methyl (4*S*)-3-(4-chlorophenyl)-4-methyl-1-[*N*-(4-chlorophenyl)carbamoyl]-2-pyrazoline-4-carboxylate had higher inhibitory activity against Ca^{2+}-uptake and higher insecticidal activity than the *R*-isomer, but the difference was greater in the Ca^{2+}-uptake system.

Keywords: *N*-Arylcarbamoylpyrazolines, calcium uptake, rat brain synaptosomes structure–activity relationship, substituent effects

1 INTRODUCTION

The *N*-phenylcarbamoylpyrazolines (Fig. 1, **I**) have potent insecticidal activity against a broad spectrum of insects.[1,2] These compounds cause complex poisoning symptoms such as convulsion and paralysis with violent tremors in insects.[3–5] These symptoms have been ascribed to the voltage-dependent inhibition of sodium channels, which has been measured in nerve preparations from insects and crayfish.[3,4,6] One of the pyrazolines (**I**, X = *para*-CF_3, Y = *para*-Cl, A = CH_3, B = $COOCH_3$; RH-3421) blocked depolarization-coupled ^{22}Na-uptake into mouse brain synaptosomes.[7] It was suggested that the compound allosterically inhibited the binding of an alkaloid activator of voltage-sensitive sodium channels to the mouse neural membrane.[8] RH-5529 (**I**: X = H, Y = *para*-Cl, A = CH_3, B = $COOCH_3$) and RH-3421 potently inhibited the spontaneous release of neurotransmitters and the transmitter-releasing effects of sodium channel activators in the guinea pig cortex.[9] These compounds also suppressed



(**I**)

Fig. 1. General structure of *N*-phenylcarbamoylpyrazolines.

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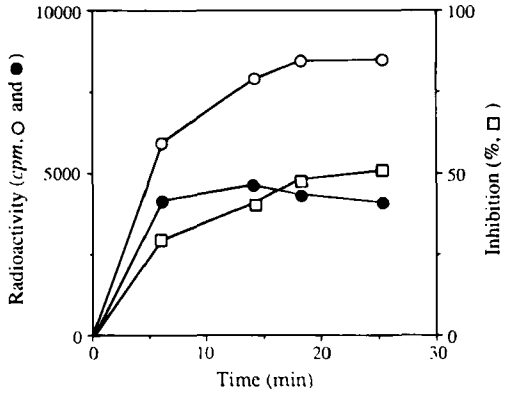


Fig. 2. Time–response relationship for $^{45}Ca^{2+}$ uptake in rat brain synaptosomes. (○) control; (●) treated with compound **1** ($1{\cdot}26 \times 10^{-5}$ M); (□) inhibition percentage for the treated samples.

potassium- and veratridine-induced increases in the synaptosomal free Ca^{2+} concentration in the absence and presence of tetrodotoxin, but not of the concentration within resting synaptosomes, indicating that the pyrazolines directly inhibit Ca^{2+} entry into the nerve endings through voltage-sensitive calcium channels.[10,11]

Twenty-six *N*-phenylcarbamoylpyrazolines have been prepared having various substituents (X) at the carbamoyl moiety (**I**: Y = *para*-Cl, A = CH_3, B = $COOCH_3$) including RH-3421 and RH-5529, and the substituent effects upon the insecticidal activity have been quantitatively analysed.[5] To clarify the substituent effects of the compounds at the target site, an in-vitro assay system had to be established. Among the nerve effects reported,[3,4,6–11] we selected ATP-dependent $^{45}Ca^{2+}$-uptake into the synaptosomes prepared from the rat brain to examine the effects of pyrazolines, because Ca^{2+} uptake is one of the basic phenomena for nerve excitation.[12–15] In this report, we show that the electronic effect is the most important feature of the substituents in governing potency. We also show the relationship between the inhibition of $^{45}Ca^{2+}$-uptake and insecticidal activity.

2 EXPERIMENTAL

2.1 Chemicals

[^{45}Ca] Calcium chloride in aqueous solution (0·37–1·50 GBq mg^{-1} Ca) and Aquasol II, a liquid scintillation counting cocktail, were obtained from Amersham International plc (Buckinghamshire, England) and DuPont-NEM (Boston, MA, USA), respectively. The test compounds listed in Table 1, except for the enantiomeric compounds **1a** and **1b**, were the same as those used earlier.[5] They were chosen as insecticidally potent compounds. Preparation of the enantiomeric compounds **1a** and **1b** is described elsewhere.[16] Adenosine 5′-triphosphate (ATP; Tris salt) and the BCA protein assay reagents were purchased from Sigma Chemical Co. and Pierce Chemical Co., respectively.

2.2 Synaptosomal preparation

Synaptosomes were prepared from the rat forebrain as described elsewhere.[12,17] Adult male Wistar rats (200–250 g) were obtained from a local supplier. They were kept in an incubator at 25°C with water and rat food for at least one day before being killed by decapitation. The forebrains were rapidly removed and rinsed in ice-cold buffer (50 mM Tris-HCl containing 0·32 M sucrose, pH 7·4; 20 ml) to remove blood and other debris. The brain was rinsed again in fresh ice-cold Tris buffer (20 ml), chopped into small pieces with scissors, then homogenised by means of 10 strokes in a glass Potter–Elvehjem homogeniser. The homogenate was centrifuged at 3000*g* for 10 min at 0°C. The supernatant was centrifuged again at 10000*g* for 20 min to obtain light yellowish pellets as the synaptosomal preparation. The pellets were resuspended in the same buffer, and the protein concentration was assayed using the BCA reagents.[18] The synaptosomal suspension was used for $^{45}Ca^{2+}$ uptake within 1 h after preparation.

2.3 $^{45}Ca^{2+}$ uptake

The $^{45}Ca^{2+}$ uptake into the rat brain synaptosomes was measured as described.[17] An aliquot (100 µl) of the synaptosomal preparation containing about 250 µg of protein was suspended in incubation buffer (3 mM magnesium chloride, 50 mM Tris-HCl, pH 7.4; 1·9 ml). To this 3 µl of methanol alone or a methanol solution of each test compound was added, and the mixture was incubated for 10 min at 0°C. The uptake reaction was started by adding 40 µl of a mixture of ATP (Tris salt) and $^{45}Ca^{2+}$ (50000–80000 cpm) to a final concentration of 2 mM ATP and 0.1 mM calcium chloride. The tubes containing the reaction mixture were quickly transferred to a water bath (37°C) and incubated for 14 min to allow $^{45}Ca^{2+}$ uptake, unless otherwise noted. The mixture was immediately filtered through a 0·45-µm filter (Nihon Millipore Ltd., Yonezawa, Japan) followed by three washes with ice-cold buffer (3 mM magnesium chloride, 100 mM sodium chloride and 50 mM Tris-HCl, pH 7·4). The filters were dissolved in Aquasol II and radioactivity was measured in a liquid scintillation counter (Wallac System 1000, Finland). The radioactivity was corrected for nonspecific binding by boiling samples of the synaptosomal material for 1 min before adding the mixture of ATP and $^{45}Ca^{2+}$. From the concentration–response curve, the half effective concentration, I_{50}(M), was calculated by probit analysis.[19,20]

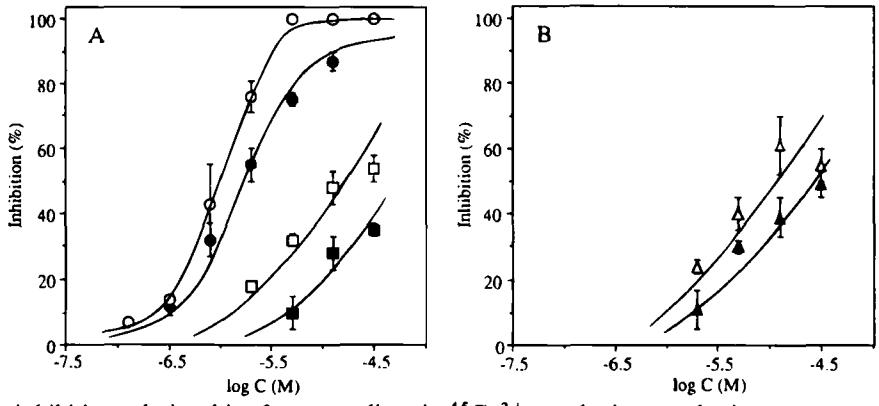


Fig. 3. Concentration-inhibition relationships for pyrazolines in $^{45}Ca^{2+}$ uptake into rat brain synaptosomes. (a) for racemic compounds (□) **1**, (■) **4**, (●) **5**, (○) **6** and (b) for the (△) *S*-isomer and (▲) *R*-isomer of compound **1**.

3 RESULTS

3.1 Effects of pyrazolines on $^{45}Ca^{2+}$ uptake

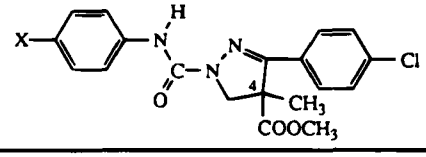
Fig. 2 shows the time-course of the incorporation of $[^{45}Ca^{2+}]$ into the rat brain synaptosomes. Incubating the synaptosome suspension with $[^{45}Ca^{2+}]$ and ATP resulted in an initial rapid phase of uptake up to about 14 min. At about 18 min after the start of incubation, the uptake reaction reached a maximum, being about 12% of $[^{45}Ca^{2+}]$ available in the buffer. When the synaptosomes were firstly incubated with racemic compound **I** at $1{\cdot}26 \times 10^{-5}$ M, the uptake was inhibited. The percentage inhibition shown in Fig. 1 was calculated according to eqn (1),

$$\text{Inhibition (\%)} = [1 - (T - N)/(C - N)] \times 100 \quad (1)$$

where T and C are the counts for treated and non-treated (control) samples, respectively, and N represents

TABLE 1

Insecticidal and Synaptosomal Activities of Methyl 3-(4-Chlorophenyl)-4-methyl-1-[*N*-(4-substituted phenyl)carbamoyl]-2-pyrazoline-4-carboxylates



Compound			Biological activities					Substituent parameters	
			log(1/I_{50})			log(1/MLD)			
No.	X	Configuration[a]	Obsd	Calcd[b]	Calcd[c]	Obsd[d]	Calcd[e]	σ	σ_R
1	Cl	*RS*	4·71	4·84	4·99	9·34	9·48	0·23	−0·25
2	Br	*RS*	4·87	4·84	4·99	9·69	9·52	0·23	−0·25
3	I	*RS*	4·75[f]	5·08	4·90	9·54	9·49	0·18	−0·16
4	OC_2H_5	*RS*	4·20[f]	4·01	4·12	9·34	9·33	−0·24	−0·57
5	NO_2	*RS*	5·75	5·76	6·02	9·86	9·78	0·78	0·10
6	CF_3	*RS*	6·04	5·78	5·57	9·90	9·86	0·54	0·11
7	OCF_3	*RS*	5·49	5·50	5·22	9·49	9·70	0·35	0·00
1a	Cl	*R*	4·55[f]	[g]	[g]	8·76	9·43	0·23	−0·25
1b	Cl	*S*	5·12	[g]	[g]	9·70	9·60	0·23	−0·25

[a] At carbon 4 of the pyrazoline ring.
[b] From eqn (2).
[c] From eqn (3).
[d] From Ref. 5 for compounds **1–7** and from Ref. 16 for compounds **1a** and **1b**.
[e] From eqn (4).
[f] Estimated value by extrapolation, because of the limited solubility.
[g] Cannot be calculated, because the respective equations were derived only from racemic compounds.

the nonspecific binding. The inhibition was time-dependent. It was only about 30% after a 7 min incubation, and increased to about 50% after 18 min. Because the synaptosomal preparation was not very stable and did not take up [Ca^{2+}] ions steadily during a long incubation, 14 min was selected as an optimal incubation period to evaluate the inhibitory potency of the compounds.

Inhibition of Ca^{2+} uptake by the test compounds was concentration-dependent. The concentration–response curves for some compounds are shown in Fig. 3. At 5×10^{-6} M, compound **6** (RH-3421) almost completely inhibited the uptake and compound **5** inhibited it by about 70% (Fig. 3(a)). The *S*-isomer of the Cl derivative (compound **1b**) tended to be more inhibitory than the *R*-isomer (compound **1a**) (Fig. 3(b)). Even though the standard deviation for each point was not very small, the I_{50} (M) value was calculated for each compound. The $\log(1/I_{50})$ values for the compounds tested are listed in Table 1. During the course of this study, we noticed that, among those prepared previously,[5] only the insecticidally potent pyrazolines gave a definitive $\log(1/I_{50})$ value, so seven racemic compounds are chosen besides the enantiomers.

3.2 Quantitative analysis of substituent effects upon the inhibition of Ca^{2+} uptake

Among those tested, the most potent compound was **6**, followed by **5** and **7**. The potency of compound **2** was close to that of compound **1**. Variations in the potency were analysed using substituent parameters to formulate eqns (2) and (3) as the most satisfactory equations.

$$\log(1/I_{50}) = 2{\cdot}603\sigma_R + 5{\cdot}495 \qquad (2)$$
$$(0{\cdot}243) \quad (0{\cdot}932)$$
$$n = 7 \quad s = 0{\cdot}214 \quad r = 0{\cdot}955 \quad F_{1,5} = 51{\cdot}59$$

$$\log(1/I_{50}) = 1{\cdot}862\sigma + 4{\cdot}565 \qquad (3)$$
$$(1{\cdot}035) \quad (0{\cdot}432)$$
$$n = 7 \quad s = 0{\cdot}314 \quad r = 0{\cdot}900 \quad F_{1,5} = 21{\cdot}40$$

In these and the following equations, n is the number of compounds, s is the standard deviation, r is the correlation coefficient, and F is the value of the ratio between regression and residual variances. The figures in parentheses are the 95% confidence intervals of the regression coefficients. σ is the Hammett constant which represents the electron-withdrawing ability of the substituent. σ_R is the resonance component of the ordinal σ value.[21] Equations (2) and (3) suggest that the activity increased by introducing electron-withdrawing substituents. The inhibitory activities calculated by eqns (2) and (3) are listed in Table 1.

4 DISCUSSION

Doherty *et al.* have evaluated the inhibitory activity of a set of pyrethroid insecticides in the ATP-dependent Ca^{2+} uptake into rat brain synaptosomes[17] and crayfish nerve homogenates.[22] They established a parabolic relationship between the inhibition index for Ca^{2+} uptake and the log P values of the compounds. Even though the test compounds and their evaluation method differed from ours, we did not find such a relationship for the pyrazoline compounds. Instead, the electron-withdrawing effect, σ, seemed to be the best to correlate the Ca^{2+}-uptake inhibition, as shown in eqns (2) and (3).

Addition of hydrophobic and/or steric parameters, which were significantly involved in the correlation equation for the insecticidal activity,[5] to eqns (2) and (3) did not improve these equations. Regardless of the σ or σ_R term, these electronic effects suggest that the Ar-NHCO-moiety of the compounds has an important role in the activity as suggested in our previous papers on insecticidal activity.[5,16] The reason why σ_R gave a more significant equation is not clear.

The increasing order of compounds in Ca^{2+}-uptake inhibition in terms of $\log(1/I_{50})$ was similar to that of insecticidal activity, log(1/MLD),[5] where MLD is the minimum lethal dose against American cockroaches (*Periplaneta americana* L.; Table 1). The relationship between these two activities is shown in Fig. 4 and was quantitatively analysed only for the racemic compounds **1**–**7** to yield eqn (4).

$$\log(1/\mathrm{MLD}) = 0{\cdot}287 \log(1/I_{50}) + 8{\cdot}126 \qquad (4)$$
$$(0{\cdot}229) \quad (1{\cdot}179)$$
$$n = 7 \quad s = 0{\cdot}143 \quad r = 0{\cdot}822 \quad F_{1,5} = 10{\cdot}40$$

Even though the number of compounds is small and the slope of the $\log(1/I_{50})$ term is much smaller than unity,

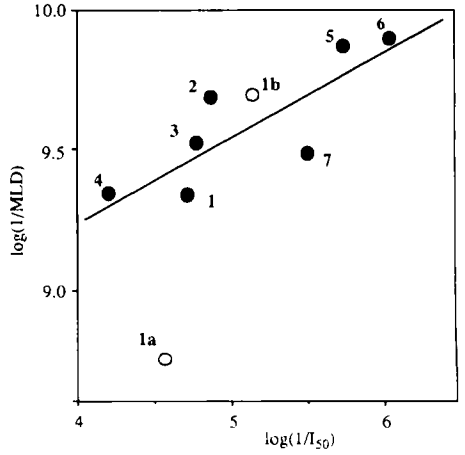


Fig. 4. Relationship between $^{45}Ca^{2+}$-uptake inhibition and insecticidal activities. Numbers correspond to those in Table 1. Compounds **1a** and **1b** were not included in deriving the regression line.

eqn (4) may mean that the higher the inhibitory activity of the ATP-dependent Ca^{2+}-uptake, the higher is the insecticidal activity. There are quite a few examples to correlate the in-vivo and in-vitro activities of a set of compounds by considering their hydrophobicity, log *P*, as the transport factor.[23–25] However, the addition of a log *P* term did not improve the quality of eqn (4). This may be due to the fact that the log *P* values did not change much among these seven compounds.

The *S*-isomer (**1b**) was more active than the *R*-isomer (**1a**) in both activities (Table 1). However, the difference in Ca^{2+}-uptake activity between these two compounds was not so large as that in the insecticidal activity. Equation (4) seems to predict well the insecticidal activity of the *S*-isomer (**1b**) but not that of the *R*-isomer (**1a**) (Table 1). This may mean that the stereospecificity of the Ca^{2+}-uptake system in rat brain is different from that in insects. Particularly, the mammalian system may be less sensitive to the *R*-isomer than the insect system.

In conclusion, our findings indicate that pyrazolines are likely to have a new site of action related to energy-dependent Ca^{2+}-uptake into the nerve terminals. Along with the reported inhibitory effects upon voltage-sensitive calcium channels,[10,11] this action may partly contribute to the neurotoxicity of mammals and/or insects. The ATP-dependent $^{45}Ca^{2+}$-uptake system in the synaptosomes seems to be convenient and useful for structure–activity and mode-of-action studies of insecticides.

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